Deep trees: Woodfall biodiversity dynamics in present and past oceans

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Marine deposits of sunken wood provide an important habitat for deep-sea biota, including an extensive wood-endemic invertebrate fauna. Polyplacophoran molluscs (chitons) are generally rare in deep-sea systems but are found in comparatively high diversity and abundance on tropical sunken wood. A new time-calibrated phylogeny for the predominantly deep-sea order Lepidopleurida shows the chiton lineages found in sunken wood habitats do not comprise a single clade or radiation, but represents a minimum of three independent radiations in the Pacific alone. Most marine invertebrate groups diversified in the deep sea following the end Cretaceous extinction event; by contrast, sunken-wood chitons may have persisted in these habitats for longer than other animals. Fossil chitons from the early Carboniferous (ca. 350 Mya) have strong similarities to modern wood-endemic taxa, yet the common ancestor of living Lepidopleurida occurred much later in the Triassic and did not apparently rely on woodfall. Clades within Lepidopleurida that occupy wood habitats in the tropical Pacific probably arose in the Jurassic, which corresponds to evidence from the fossil record, but with an additional separate colonisation more recently in the early Paleogene. Wood-endemic chiton species encompass multiple independent evolutionary origins of co-occurring wood species, and these separate lineages correspond to differences in micohabitat and feeding strategies. These patterns demonstrate the ongoing evolutionary linkages between terrestrial and deep marine environments, and the opportunistic adaptations of deep-sea organisms.

Introduction

New data and perspectives have repeatedly overturned historical assumptions about deep-sea biodiversity. The *Challenger* expeditions provided the first records of life on sunken wood (Murray, 1895), early evidence against the long-held idea of an azootic abyss. More recently, the idea that ancient relics or living fossils dominate deep-sea faunas has been challenged by molecular data (Vrijenhoek, 2013). Time-calibrated phylogenetic reconstructions of animal clades in deep sea hydrothermal vents, seeps, and organic falls, have consistently recovered evidence of Cenozoic radiations and colonization events (e.g. Lorion et al., 2013; Roterman et al., 2013; Johnson et al. 2010; Taylor et al., 2014). Woodfall faunas have been relatively under-studied compared to other chemosynthetic-based ecosystems. These habitats have usually been examined as a putative 'stepping stone' to other chemosynthetic oases (Distel et al. 2000; Bienhold et al. 2013), either literally in animals that can occur on multiple organic substrata (Lorion et al., 2009), or as the habitat of early-diverging lineages within a clade (Lorion et al., 2013). Wood-falls are globally distributed and host significant biodiversity, and these faunas are important in their own right to past and present deep-sea biodiversity.

Polyplacophoran molluscs (chitons) are abundant on sunken wood, especially in the tropical Pacific (Sirenko, 2001; Sigwart and Sirenko, 2011). Chitons are a classic 'living fossil' taxon, which strongly resemble their fossil counterparts from over 300 million years ago (Sirenko, 2006). The unusual, wood-endemic clade Ferreiraellidae has shell valves that are similar to a fossil *Glauphurochiton carbonarius* from the early Carboniferous ca. 350 million years ago (Sirenko, 1997). This would seem to conflict with the pattern of Cenozoic radiations into the deep-sea. Alternatively, this conventional understanding of polyplacophoran evolution might belie the impacts of past ocean anoxia and the extinction events that apparently shaped other deep sea lineages (Vrijenhoek, 2013).

Chitons are broadly divided into two clades: Chitonida, a predominantly shallow-water group, and Lepidopleurida, which contains the majority of deep water taxa and those known from sunken wood habitats (Okusu et al., 2003; Sirenko, 2006; Sigwart

et al., 2013). Recent molecular studies of Lepidopleurida showed some evidence of multiple wood-colonisation events in the evolution of this major deep-sea clade (Sigwart et al., 2011; Duperron et al., 2013), although that was not a major conclusion of either study. Among chitons that live on sunken wood, some are wood-digesting species and others graze on surface biofilms (Duperron et al., 2013). Lepidopleuran chitons are considered plesiomorphic and they live in both shallow waters and the deep sea. This clade provides an excellent context to examine questions of temporal dynamics in colonization of deep sea habitats and particularly sunken wood. The purpose of this study was to estimate divergence times of lineages within Lepidopleurida that inhabit sunken wood through a time-calibrated phylogenetic analysis.

Methods

The present analysis combined all available published sequences from previous studies that have included species in Lepidopleurida (Okusu et al., 2003; Passamaneck et al., 2004; Kelly et al., 2007; Sigwart et al., 2011; Duperron et al., 2013; Lobo et al., 2013; Layton et al., 2014). Six additional species from Chitonida were used as outgroups: *Callistochiton antiquus*, *Chaetopleura apiculata*, *Ischnochiton comptus*, *Acanthochitona crinita*, *Cryptoplax japonica*, *Mopalia muscosa*, using previously published sequence data. This analysis used from three standard markers, aligned and truncated to maximise overlap: COI (657 bp), 18S (1795 bp) and 28S (774 bp). All sequences were quality checked, and subjected to BLAST search to exclude contamination, resulting in a final total of 96 terminals (animal specimens) covering 44 species (Appendix 1).

The monophyly of Lepidopleurida is uncontroversial, and supported by numerous morphological synapomorphies (Sigwart et al., 2014). Previous molecular phylogenetic studies have had relatively limited taxon sampling from Lepidopleurida (e.g. Okusu et al., 2003; Stoeger et al., 2013), and for completeness, initial analyses directly tested lepidopleuran monophyly by including comparative sequences of both monoplacophorans and aplacophorans as outgroups. These preliminary analyses uniformly resulted in a monophyletic Lepidopleurida and had no influence on the ingroup topology, thus full analyses did not include any taxa outside Polyplacophora.

Proximate outgroups (from the siter clade Chitonida) are most appropriate to resolve ingroup lepidopleuran relationships.

Pairwise distances of COI were calculated with the software MEGA 6.06 (Tamura et al., 2013). Appropriate substitution models for each of the three partitions were determined by log-likelihood and Akaike Information Criteria using JModelTest (ver 2.1.7); results for each partition were equivocal between a symmetric model (SYM+I+Γ), or general-time reversible model (GTR+I+ Γ), which has been reported for other chitons (Sigwart et al., 2013). Preliminary Bayesian analyses using both models found no differences in resultant topology.

All phylogenetic analyses were implemented via the CIPRES Science Gateway (Miller et al. 2010). Phylogenetic analyses using Bayesian inference were performed with MrBayes (ver 3.2.1) on the partitioned dataset using the SYM+I+ Γ for each (COI, 18S, 28S), combining two runs of four chains, in 100 million generations, sampling one tree every 1000 generations, temperature 0.2, discarding *a priori* 20% burn-in, i.e. resulting in a consensus tree summarizing 150,000 retained trees. Node support vales were recorded by default as bipartition posterior probabilities.

The MrBayes consensus tree was then used as the starting tree for time-calibrated phylogenetic analyses performed in BEAST. The tree file was re-rooted and reformatted in R (R Core Team, 2015) to ensure that zero-length branches were not read as polytomies by using the 'multi2di' command from the 'ape' library (Paradis et al., 2004).

For comparison, phylogenetic analyses using maximum likelihood were performed with RaXML (Stamatakis, 2014) with the data partitioned into three units corresponding to the three markers; as a symmetrical model is not available, GTR+I+ Γ was used as the substitution model for the full dataset. Bootstrap support values were determined via the combined likelihood search and bootstrapping protocol, allowing RaXML to determine the optimum length.

The time-calibrated phylogeny was determined in BEAST (Drummond et al., 2012) using three calibration points based on the chiton fossil record. Based on previous

analyses, Leptochitonidae *sensu stricto* contains two genera (*Lepidopleurus* and *Leptochiton* s.s.) and is restricted to the Mediterranean and northeast Atlantic (Sigwart et al., 2011). The oldest fossil in this grouping is *Lepidopleurus virgifer*, from the Oligocene / Miocene of Europe, 23 Mya. The type locality of this species is from the Oligocene (Chattian?) of Gienberg, near Waldböckelheim, Germany (Sandberger, 1858–1863), but other closely allied material is known from the lower Miocene (Dell'Angelo and Palazzi, 1989), the selected calibration date was conservatively placed at the Oligocene / Miocene boundary. This date also corresponds approximately to the formation of the modern Mediterranean basin (Steininger and Rögl, 1984; Bunje and Lindberg, 2007). Therefore this calibration point was used as a proxy for the split between *Lepidopleurus* (restricted to the Mediterranean) and *Leptochiton* s.s. (in the Mediterraean and NE Atlantic). The oldest fossils in a living genus of Lepidopleurida are two species, *Leptochiton davolii* and *L. reiectus* from the Triassic of Italy, 231 Mya (Laghi, 2005). This date was therefore used as the calibration for the radiation of the living members of Lepidopleurida. Another fossil chiton, *'Hoareus?' dalriensis* from the Carboniferous of Scotland has been suggested to be a member of *Leptochiton* (Sirenko, 2013) or at least Leptochitonidae *sensu lato* (Özdikmen, 2010), but it appears to be more similar to other Carboniferous genera so was excluded from consideration here. The oldest chiton with slit insertion plates and therefore attributable to Chitonida is *Ochmazochiton comptus*, from the Permian of West Texas, *circa* 276.5 Mya (Hoare and Smith, 1984). As Chitonida and Lepidopleurida are sister-taxa, the crown group Lepidopleurida must have radiated subsequent to the origination of Chitonida; the date of 276.5 Mya is a reasonable estimate for the split between Chitonida + Lepidopleurida, the root age in the present anaylsis.

Analyses in BEAST were implemented on the three-partition dataset using the SYM+I+G substitution model applied separately to each partition (as in MrBayes anlyses), identifying taxa in Lepidopleurida as monophyletic with a calibrated origin and Leptochitonidae *s.s.* as monophyletic with a calibrated origin date. The three calibration points (Leptochitonidae *s.s.*, Lepidopleurida, and root age) were each set with a gamma prior shape 5.0, and scale 2.7, based on previous analyses for this class in larger pan-Molluscan analyses (Stoeger et al. 2013), and the offset for each node set as the upper (youngest) extent of the relevant geological Epoch. The model

implemented a relaxed lognormal molecular clock for the total combined dataset, with a Yule speciation tree prior. Both the speciation rate and the ucld mean used a relaxed gamma prior with initial median value of 0.004, shape 0.001 and scale 1000. The analysis was run for 500 million generations, sampling 1 tree every 5000 generations to minimize autocorrelation, discarding a 20% *a priori* burn-in. Thus the final results reported represent is a summary of 80,000 retained trees.

Transitions between depth ranges were analysed via ancestral state reconstructions in Mesquite (Maddison and Maddison 2015) using parsimony and Maximum Likelihood to calculate most parsimonious states and proportional likelihoods for character states at each node of the time-calibrated tree produced in BEAST. The midpoint of the depth range (median depth) of the collecting data for each individual specimen was coded as a continuous character (Appendix 1). Reconstructed ancestral states for were subsequently summarised into broader ranges.

Results

The same topology was recovered from all phylogenetic analyses herein, with the exception of the position of Ferreiraellidae. Within Lepidopleurida, *Hanleya nagelfar* was the earliest-braching taxon, separate to all other ingroup taxa. The rest of the sampled taxa were divided into Protochitonidae + Leptochitonida s.s. opposed to all other sampled taxa. And that subset was subdivided further into a large wood-dwelling clade, sister to a grouping 'Clade I' of other worldwide species in *Leptochiton* s.l. and *Parachiton*. The position of Ferreiraellidae (*Ferreiraella* spp.) was equivocal between Bayesian analyses (Figure 1) and Maximum Likelihood (results not shown) where *Ferreiraella* was sister to 'Clade I'. Both topologies had only moderate support, with clade credibility values of 58 (MrBayes, Ferreiraellidae+wood-dwelling taxa) or 50 (RaXML Ferreiraellidae+Clade I; not shown). The position of Ferreiraellidae must be considered equivocal (Figure 1).

The two most densely-sampled species, *L. rugatus* and *L. boucheti*, show divergence patterns indicative of cryptic species. *Leptochiton rugatus* was clustered according to geographical regions, with specimens from the eastern Pacific (California and Mexico) sister to all others, including records from across the north Pacific (Russia,

Canada, and the northern U.S.) as well as one deeper-water record from California identified as *L.* cf. *giganteus* (Figure 1). All of the sampled specimens of *L. boucheti* were from Vanuatu; they resolved within a clade of other '*Leptochiton'* spp. from Vanuatu that also included two samples from the Philippines (*L. samadiae* B, *L. deforgesi* B) (Figure 1, Appendix). The pairwise distances for COI among *L. boucheti* specimens were very low, except for a single specimen *L. boucheti* D, which was over 29% different from conspecifics; excluding that single sample and excluding identical sequences with a difference of 0 the mean pairwise distance for *L. boucheti* was 0.57%.

The time-calibrated analysis was based on three prior calibration points (Lepidopleurida / Chitonida, crown group Lepidopleurida, and Leptochitonidae s.s.), and resolved posterior estimates for those nodes very similar to the prior dates for the two deeper nodes: 284 My for the split between Lepdiopleurida and Chitonida (95% highest posterior density interval [275.0, 295.1], i.e. Permian, Cisuralian), and 233 My for crown-group Lepidopleurida (95% HPD interval [223.4, 245.1], Middle Triassic to early Late Triassic). The more shallow calibration point had an earlier posterior estimate, 44 My with a broad 95% HPD interval entirely deeper than the prior starting value at 23 My (95% HPD interval [28.1, 62.0], Palaeogene).

The wood-dwelling species sampled do not form a monophyletic clade. Accepting the monophyly of the majority of wood-dwelling taxa (i.e. including Ferreiraellidae) suggests an origination of that habitat preference at 162 My [117.2, 204.8], in the Jurassic (Figure 1). The wood-dwelling species *L. foresti* was not part of that clade and diverged from the most closely related sampled taxon around 57 My ago [8.1, 158.7], though that range was poorly resolved.

These most closely-related clusters of conspecifics had common ancestors in the early Neogene, both wood-dwelling taxa and not: *L. boucheti* (samples E-T) diverged 19 Mya [4.1,73.0], and *L. rugatus* 2 (high latitude N Pacific specimens) had a common ancestor 24 Mya [4.1,88.0]; but *L. rugatus* 1 (specimens from the Pacific of California and Mexico) diverged in the Palaeogene, at 47 My [11.1,188.8], although those dates all fell in effectively equivalent ranges (Figure 1).

Parsimony-based reconstructions of ancestral state for habitat depth indicated an ancestral condition of moderate depth (400-510 m), with divergences to both deeper (*Hanleya nagelfar*) and shallower (Leptochitonidae + Protochitonidae, Clade I) radiations (Figure 1).

Discussion

Major Cenozoic events that influenced other deep marine clades, such as the end Cretaceous or the Paleocene-Eocene Thermal Maximum (PETM), do not seem to have had any marked impact on chiton diversification. Lepidopleuran chitons are an ancient but not a static clade, and many diversification events during the Cenozoic show transitions to novel habitats and depths. The persistence of these 'living fossils' has been achieved through opportunism.

New results here show a major radiation of chitons on sunken wood in the Jurassic, but with several independent colonization events over the history of the group. The oldest fossil evidence of woodfall faunas comparable to the modern deep-sea biota is from the Middle Jurassic of Poland, assemblages which lack xylophagain bivalves but do contain fossil lepidopleuran chitons (Kaim, 2010). Another fossil chiton from the Upper Jurassic Russian Sea, *L. shapovalovi*, was associated with wood fragments and other non-obligate wood species (Sirenko 2013). Chitons were seemingly early inhabitants of modern-type sunken wood in the Mesozoic and remained there throughout other global environmental perturbations. Establishment of the living lineages on wood in the Jurassic is substantially earlier than the Cenozoic timeline for colonization of chemosynthetic habitats (including vents, seeps, and also organic falls) in other molluscs (Vrijenhoek, 2013).

Three taxa (*L. laurae, L.* cf. *pergranatus*, '*Leptochiton'* sp.) from other chemosynthetic habitats (cold seeps) all originated after the Paleocene-Eocene Thermal Maximum, and are related to other species from non-chemosynthetic habitats. These may be 'opportunistic' filling of available niches in cold seeps following the same pattern as other marine invertebrate clades colonising seeps after the anoxic/dysoxic event associated with the PETM (Vrijenhoek, 2013). This may not be a direct causal link, as the influence of oceanic anoxic on early history of the

modern deep-sea fauna likely depends on specific biological groups (Jacobs and Lindberg, 1998; Smith and Stockley, 2005).

Chitons are not common constituents of hydrothermal vents or other geothermal sites, and evolution of deep-sea marine diversity clearly does not proceed in a linear trajectory from organic falls toward hydrothermal vents. In polychaetes, closely-related specialists from organic-rich habitats in deep and shallow ecosystems and hydrothermal vents have indicated multiple invasions of deep-sea habitats from shallow ancestors (Wiklund et al. 2012). In the case of bathymodiolin mussels there is evidence of a radiation originating in wood faunas, with at least four independent invasions of vent habitats (Thubaut et al. 2013). In mussels, and in chitons, the wood fauna may serve as a 'biodiversity generator' or reservoir (Smith et al. 2015). But the evidence from chiton evolutionary dynamics indicates flexibility both in depth and specialism.

Lepidopleurida is characterised as a deep-sea group, though a number of its species occur in coastal waters and even the intertidal (Sirenko 2006). The present phylogenetic analysis used different analytical approaches and additional taxa, yet supports the previous published phylogeny for Lepidopleurida (Sigwart et al. 2011), suggesting the general topology is an accurate reconstruction of their radiation. The lepidopleuran ancestor is inferred to have occurred at moderate depth (around 500 m), with subsequent excursions either deeper (*Hanleya*) or more shallow (Clade I + Leptochitonidae *s.s.*). Some fossil evidence also supports *in situ* diversification of deep-sea lineages (Thuy et al. 2014). This contrasts with the coastal origination of other deep-sea benthic mollusc clades, where shallow radiations and repeated invasion of deeper-water environments are more typical (e.g. Taylor et al., 2013; Williams et al. 2013). Other invertebrates have been found to include shallow descendents of deep-sea lineages (Linder et al. 2008), challenging the idea of the deep-sea invasion from shallow water as a consistent pattern (Jablonski et al. 1983; Vrijenhoek, 2013).

Species-level diversity is probably underestimated among living lepidopleuran chitons (Kelly et al. 2007). There is a well-established suite of characters that enable the reliable separation of species, and new fossil and living chiton species are regularly

discovered and described. The genus *Leptochiton* is paraphyletic (Sigwart 2009a). Although morphology can separate species, and sometimes groups of species, there are no evident unifying morphological features that correspond to molecular clades within *Leptochiton* (Sigwart et al. 2011). This confounds systematic revision for this group and also presents a barrier to integrating fossil evidence to the broader reconstruction of chiton and wood-fauna evolution and diversity.

There are several fossil chitons from sunken wood attributed to *Leptochiton* sensu lato. *Leptochiton lignatilis* from a Miocene fossil woodfall assemblage from north Italy was compared to several tropical wood-dwelling species (Bertolaso et al. 2015). Based on its age and paleogeography, the fossil *L. lignatilis* is at least as likely to be related to Leptochitonidae s.s. from the Mediterranean and north-east Atlantic. Separately, fossil *Leptochiton* specimens have been recorded in at least five Eocene–Miocene woodfall assemblages in Washington State, from material that was originally attributed to a Recent species *L.* cf. *alveolus* (Squires & Goedert 1995, Kiel & Goedert 2006, J.L. Goedert pers. comm.). From its geography, that species most likely has affinities to Lepidoplerida 'Clade I' (Figure 1). These two fossils (*L. lignatilis* from Italy, *L.* cf. *alveolus* from Washington) could represent additional novel colonisations of wood-falls, potentially expanding the history of wood-fall occupation to two more clades within Lepidoplerida: Leptochitonidae *s.s.* and Clade I. A frustrating level of potential homoplasy in key taxonomic characters prevents resolution of these important questions.

Unusual morphological features are taxonomically important in this morphologically homogeneous group. Morphological comparisons, in combination with an apparent association of a fossil with plant remains, led Sirenko (1997; 2004) to suggest that the Carboniferous fossil *Glauphurochiton carbonarius* was potentially an ancestral lineage to the living genus *Ferreiraella*. But the Carboniferous pre-dates fossil assemblages most similar to modern woodfall faunas, and the fossil communities that included *Glauphurochiton carbonarius* may not be true fossils of deep-sea sunken wood (Kaim, 2010). It seems more parsimonious that *Glauphurochiton* and *Ferreiraella* are morphologically convergent. The genus *Ferreiraella* is known from several globally distributed species including the eastern Pacific and also the Caribbean (Sirenko, 1997; 2004); inclusion of additional taxa would be necessary to

resolve the equivocal topological position of this clade, but *Ferreiraella* probably represents an independent radiation on woodfall.

Despite a long history of association with sunken wood, the evolution of chitons includes several independent colonisations of wood throughout the Mesozoic and Cenozoic. Several species in the Philippines co-occur on sunken wood that represent two different wood-colonisation events (clade I: *L. foresti*, versus: *L. samadiae*, *L. vietnamensis*, *Nierstraszella* spp.). The consistency of this topological result (Sigwart et al. 2011), and the distinctive shell morphology of *L. foresti* (Sigwart and Sirenko 2011) provide evidence that it represents a completely independent evolution of the wood-dwelling lifestyle even in one geographic area.

The genus *Nierstraszella* is a wood-endemic clade, known from only two species (Sigwart 2009b). It co-occurs with a number of other species on wood in the tropical Pacific (Sigwart and Sirenko 2011). *Nierstraszella lineata* hosts wood-digesting bacteria in its gut, in contrast to *L. boucheti*, which lives on wood but has a very different microflora and apparently digests biofilms (Duperron et al. 2013). Extending these phylogenetic results and the separation of diet, suggests that species allied with Nierstraszellidae (*L. longisetosus*, *L. saitoi*, *L vietnamensis*, *L. vaubani*) are wood-digesting. All of these taxa found on woodfalls have wood particles commonly found in the gut (e.g. Sirenko 2001), but an ability to digest of the wood itself has so far only been demonstrated in *Nierstraszella lineata* (Duperron et al. 2013). Further examinations of the microflora is required to determine whether this bacterially mediated mode of feeding evolved once (in Nierstraszellidae or an ancestor) or perhaps multiple times.

Nierstraszella has an origination estimated approximately in the Late Cretaceous (Figure 1), which is coincident with the availability of angiosperm wood (Willis and McElwain 2002). Most of these species, in the clades containing *N. lineata* and *L. boucheti* live predominantly on angiosperms, particularly palm (Pante et al., 2012). Direct nutritional dependency on wood separates these chitons from other animals that use wood for substrate. Suspension feeders such as mussels may be more able to transition to other organic falls or any suitable substrate. But perhaps the majority of

chiton lineages found on sunken wood are grazers that would equally be able to transition to other habitats.

Lepidopleuran chitons have occupied deep sea woodfalls since the origin of modern sunken-wood communities starting in the Jurassic. Throughout the Cenozoic, this group radiated into both deeper and more shallow habitats, and colonised chemosynthetic habitats (sunken wood and cold seeps) multiple independent times. Despite morphological consistency over extended periods, these 'living fossils' remain flexible and opportunistic in occupying available deep sea niches.

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Figure captions.

Figure 1. Time-calibrated phylogeny of relationships within Lepidopleurida, from combined analysis of three standard markers (18S, 28S, COI gene regions), implemented on Bayesian inference using BEAST. Node support values show posterior probabilities (PP) from Bayesian inference, but are simply excluded for minor branches that are generally over 95%. Shading of branches indicates the habitat depth from dark (> 1750 m) to pale (< 350 m), with ancestral states inferred via parsimony. Background shading indicates parsimony reconstruction of ancestral habitat states, for wood dwelling (green) and other chemosynthetic (gold) habitats. As the position of Ferreirallidae is considered equivocal the ancestral origination of sunken-wood lifestyles are indicated with dashed lines to show two alternative equal scenarios. Error bars show highest posterior density intervals for important divergence points.